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Human Ca^{2+}/Calmodulin-dependent Protein Kinase Kinase β Gene Encodes Multiple Isoforms That Display Distinct Kinase Activity*

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Ca^{+2}/calmodulin-dependent protein kinases (CaMKs) are activated upon binding of Ca^{+2}/calmodulin. To gain maximal activity, CaMK I and CaMK IV can be further phosphorylated by an upstream kinase, CaMK kinase (CaMKK). We previously isolated cDNA clones encoding human CaMKK β isoforms that are heterogeneous in their 3′-sequences (Hsu, L.-S., Tsou, A.-P., Chi, C.-W., Lee, C.-H., and Chen, J.-Y. (1998) *J. Biomed. Sci.* 5, 141–149). In the present study, we examined the genomic organization and transcription of the human *CaMKK β* gene. The human *CaMKK β* locus spans more than 40 kilobase pairs and maps to chromosome 12q24.2. It is organized into 18 exons and 17 introns that are flanked by typical splice donor and acceptor sequences. Two major species of transcripts, namely the β1 (5.6 kilobase pairs) and β2 (2.9 kilobase pairs), are generated through differential usage of polyadenylation sites located in the last and penultimate exons. Additional forms of *CaMKK β* transcripts were also identified that resulted from alternative splicing of the internal exons 14 and/or 16. These isoforms display differential expression patterns in human tissues and tumor-derived cell lines. They also exhibit a distinct ability to undergo autophosphorylation and to phosphorylate the downstream kinases CaMK I and CaMK IV. The differential expression of CaMKK β isoforms with distinct activity further suggests the complexity of the regulation of the CaMKK/CaMK cascade and an important role for CaMKK in the action of Ca^{+2}-mediated cellular responses.

Ca^{2+}, an important second messenger in eukaryotic cells, regulates many cellular processes including muscle contraction, neurotransmmiter secretion, gene expression, and cell cycle progression (1, 2). Upon stimulation, elevated intracellular Ca^{2+} mediates its effects via interaction with calmodulin (CaM),[1] and Ca^{2+}/CaM binds to and induces the activity of a wide range of regulatory proteins. The family of Ca^{2+}/CaM-dependent protein kinases (CaMKs) consists of specific enzymes, *e.g.* myosin light chain kinase, phosphorylase kinase, and the multifunctional enzymes, such as the various isoforms of CaMK I, CaMK II, and CaMK IV (3–5). The multifunctional CaMKs have been shown to be involved in regulating gene expression by phosphorylating various transcription factors. A number of documents have demonstrated that the CaMK pathway is analogous to the mitogen-activated protein kinase cascade in that it requires an upstream protein kinase, CaMK kinase (CaMKK), to phosphorylate and fully activate CaMK I and CaMK IV (6–11). CaMKK purified from pig brain phosphorylates the threonine residue localized in the "activation loop" of CaMK I (Thr^{177}) and CaMK IV (Thr^{196}), respectively, and increases their activity 20–50 times. Mutation of the Thr residue to Ala abolishes both the phosphorylation and the activation of CaMK I/CaMK IV by CaMKK (12, 13).

Recently, two distinct cDNAs were isolated encoding the rat CaMKK α and β. They share 69% homology in amino acid sequence and are localized in different regions of the brain (14–19). CaMKK α is widely distributed in neurons throughout the brain, except in the cerebellar cortex, whereas CaMKK β is relatively restricted in some neuronal populations, particularly in the cerebellar granule cells (17, 18). Like other members of the CaMK family, CaMKK is composed of an N-terminal catalytic domain and a regulatory domain at its C terminus, which contains the CaM-binding site overlapped with the autoinhibitory domain (9, 20, 21). Co-expression of CaMKK with CaMK I or CaMK IV was shown to enhance the activity of CaMK I or CaMK IV toward phosphorylation of cAMP response element-binding protein (CREB) and cAMP response element-dependent reporter gene expression in a Ca^{2+}-dependent manner (15, 19). The CaMKK/CaMK/CREB pathway was recently successfully reconstituted in the *Caenorhabditis elegans* (22). Other than CREB, CaMK IV-mediated signaling is also known to be involved in Ca^{2+}-regulated gene expression through activation of serum response factor (SRF) and activating transcriptional factor-1 (ATF-1) (23–25). Intriguingly, CaMKKs and CaMK IV have been shown to exhibit different subcellular localization in the brain. In contrast to the nuclear localization of CaMK IV, both rat CaMKK α and β are localized in the perikaryal cytoplasm, dendrites, and nerve terminals (18). The distinct subcellular expression patterns suggest the presence of a complicated mechanism for the activation of CaMK IV by CaMKK.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF287630, AF287631, AF321575-AF321578, and AF321389-AF321402.

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[1] The abbreviations used are: CaM, calmodulin; CaMK, Ca^{+2}/CaM-dependent protein kinase; CaMKK, CaMK kinase; BAC, bacterial artificial chromosome; CREB, cAMP response element-binding protein; kb, kilobase(s); PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid; GST, glutathione *S*-transferase; bp, base pair(s); nt, nucleotides; RT, reverse transcription.

The CaMKK/CaMK IV cascade has also been indicated to interact with the mitogen-activated protein kinase cascade to activate c-Jun NH_2-terminal kinase and p38 (26). CaMKK was also suggested to play a role in cell survival. The rat CaMKK α was shown to phosphorylate and activate protein kinase B (PKB) which can then phosphorylate BAD protein. The phosphorylated BAD will then bind to 14-3-3 protein instead of Bclx and, thus, prevent apoptosis (27).

We previously isolated different cDNA clones corresponding to human CaMKK β that shared more than 90% amino acid sequence homology to rat CaMKK β (28). These cDNA clones are heterogeneous at their 3′-termini. To delineate the transcription of these *CaMKK β* transcripts, in the present study we examined the genomic structure and transcription of human *CaMKK β* gene. We found that the human *CaMKK β* gene contains 18 exons that span more than 40 kb. Multiple transcripts are encoded by the *CaMKK β* gene through alternative RNA processing. The properties and expression patterns of the various CaMKK β isoforms were investigated.

EXPERIMENTAL PROCEDURES

Human Tissues, Cell Lines, and RNA Preparation—Human glioblastoma/astrocytoma U-87 MG cells and glioblastoma U-138 MG cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Human non-small cell lung cancer H-1299 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. These cell lines were maintained in a 5% CO_2 humidified chamber at 37 °C. Tissues from normal brains and brain tumors were obtained from patients who underwent surgery at the Veterans General Hospital-Taipei, Taiwan. Informed consent was obtained from each patient. Tissues were snap-frozen immediately after resection. Total RNA was prepared from pulverized tissues or cell lines using the guanidine isothiocyanate method and pelleted through a 5.7 M CsCl cushion (29). The pelleted RNA was dissolved, subjected to DNase I digestion to remove residual DNA, and stored at −80 °C for future use.

5′-Rapid Amplification of cDNA Ends—To extend the 5′ cDNA sequence, a human brain marathon cDNA library was constructed utilizing a Marathon™ cDNA amplification kit according to the manufacturer's instructions (CLONTECH, Palo Alto, CA). First strand cDNA was synthesized from 10 μg of total RNA prepared from human brain tumor tissue with a *CaMKK β*-specific primer (5′-CAACTTGACGACACCATAGGAGC-3′) followed by second-strand cDNA synthesis. The double-stranded cDNA was then amplified by PCR using an adapter primer (5′-CCATCCTAATACGACTCACTATAGGGC-3′) and the gene specific primer under the following conditions: 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min for 30 cycles. The PCR product was diluted 10-fold, and 1 μl was used as the template in the nested PCR. The nested PCR was performed using a gene-specific nested primer (5′-TCTTGCAGAGACAGCTTGCG-3′) and a nested adaptor primer (5′-ACTCACTATAGGGCTCGAGCGGC-3′) under the conditions described above except that the annealing temperature was set at 50 °C. The PCR products were ligated to a pGEM-T vector (Promega, Madison, WI). Colonies containing the *CaMKK β* cDNA fragment were scored by PCR amplification using *CaMKK β*-specific sense (5′-CTCATCCTTGAGCATCCACC-3′) and antisense (5′-TCTTGCAGAGACAGCTTGCG-3′) primers under the following conditions: 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min for 30 cycles. The positive clones were sequenced in both directions for *CaMKK β* cDNA sequences using OmniBase™ DNA cycle sequencing system (Promega).

Northern Blot Analysis—The *CaMKK* β1 and β2-specific cDNA fragments were purified from K5 and K6 clones after restriction digestion with *Sac*I (located near the 3′-termini of the *CaMKK β* cDNAs) and *Kpn*I (located within the polyclonal sites of pGEM-T) (28). The N-terminal common fragment was amplified by PCR using K6 as template with oligonucleotides 5′-AGCTCCTGGAGGCTGCATC-3′ and 5′-CCAGGCGCTGACAGTGAGCG-3′ as primers. The cDNA fragments were labeled with [α-^{32}P]dCTP by random priming using the Rediprime DNA labeling system (Amersham Pharmacia Biotech). Messenger RNAs were prepared from 400 μg of total RNA using the Oligotex™ mRNA midi kit (Qiagen Inc., Valencia, CA), separated by electrophoresis on 1.2% MOPS-formaldehyde agarose gel, and blotted to a nylon membrane (Roche Molecular Biochemicals) by capillary transfer. After UV cross-linking, blots were prehybridized in ExpressHybrid solution (CLONTECH Laboratories Inc., Palo Alto, CA) at 65 °C for 30 min. Hybridization was performed by reacting with fresh ExpressHybrid solution containing the denatured probes at 65 °C for 16 h. The blots were washed twice with 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) and 0.05% SDS at room temperature for 20 min, then washed once with 0.1× SSC and 0.1% SDS at 50 °C for 20 min. The signal was obtained by autoradiography.

λ *Genomic Library Screening*—The 1.8-kb K6 cDNA fragment and the 0.6-kb fragment obtained from 5′-rapid amplification of cDNA ends were labeled with [α-^{32}P]dCTP and used as probes to screen a human placenta λ FIX II genomic DNA library (Stratagene, La Jolla, CA). After lifting the plaques, hybridization of the filters was performed at 65 °C overnight in hybridization buffer (6× SSC, 5× Denhardt's solution, 0.5% SDS, 100 μg/ml sonicated salmon sperm DNA) containing denatured probes (1×10^6 cpm/ml). The filters were washed twice with 2× SSC and 0.1% SDS at room temperature for 20 min, and once with 0.5× SSC and 0.1% SDS for 20 min at 55 °C. Three positive clone, G5-1, G2-1, and G3-1 were isolated upon hybridization of 1×10^7 plaques. The genomic DNA fragment was excised by *Not*I and subcloned into a pBluescript-KS vector for sequencing analysis.

Purification and Sequencing of the Bacterial Artificial Chromosome (BAC) Clone—BAC clone 2283L16 was obtained by PCR screening of the BAC library D_1 (Research Genetics, Inc.) using *CaMKK β*-oligonucleotides 5′-CGTATGCTGGACAAGAACCC-3′ and 5′-TCTCGACCTCCTCTTCAGTC-3′ as primers under the following conditions: 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 3 min for 30 cycles. To prepare BAC DNA, a single colony of BAC clone was cultured in 5 ml of LB medium containing 12.5 μg/ml chloramphenicol at 37 °C overnight. The overnight culture was transferred into 500 ml of TB medium (12 g bactotrypton, 24 g bacto-yeast extract, and 4 ml glycerol in 1 liter of 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4) containing 12.5 μg/ml chloramphenicol and cultured at 37 °C for 16–20 h. The BAC DNA was isolated using the alkaline lysis method, treated with RNase A (final concentration 10 μg/ml) for 3 h at 37 °C, and precipitated with 2 M NaCl and 20% polyethylene glycol 8000. After centrifugation at 13,000 × *g* for 30 min, the DNA pellet was dissolved in 0.5 M ammonium acetate, extracted with phenol/chloroform twice, and precipitated by ethanol. After centrifugation, the DNA pellet was washed with 70% ethanol and redissolved in 100 μl of TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) buffer for sequencing analysis.

Primer Extension Analysis—The oligonucleotide 5′-GATCACTGCAACCTCTGCCTCCCAG-3′ was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase at 37 °C for 10 min, followed by heat inactivation at 90 °C for 2 min. For primer extension analysis, 2 μg of mRNA prepared from H-1299 or U-87 MG cells was annealed to the labeled primer (~ 0.1 pmol) at 50 °C for 60 min and then reverse-transcribed using the Moloney murine leukemia virus reverse transcriptase (Superscript II, 200 unit, Life Technologies, Inc.) in a 20-μl reaction mixture containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM dNTP, 20 units RNasin, and actinomycin D (final concentration 50 μg/ml). The reaction was incubated at 42 °C for 50 min followed by heat inactivation at 70 °C for 15 min. The reaction mixture was incubated with RNaseA (0.5 μg/ml) at 37 °C for 30 min and extracted twice with phenol/chloroform. The reaction product was ethanol-precipitated and analyzed on a 6% polyacrylamide sequencing gel together with a sequence ladder obtained by dideoxy sequencing of the control DNA using the OmniBase™ DNA Cycle Sequencing System (Promega).

RT-PCR Analysis of Human CaMKK β mRNAs—Five μg of total RNA prepared from U-87 MG cells, U-138 MG cells, human placenta tissue, or brain tissues were converted to cDNA by Moloney murine leukemia virus reverse transcriptase (Superscript II, 200 unit, Life Technologies, Inc.) in a 50-μl reaction mixture containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM dNTP with 0.625 μg of random primer following the manufacturer's instructions. Using cDNAs as templates, 30 cycles of PCR (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min) were performed with the following oligonucleotides as primers. The sense primer F1 (5′-CGTATGCTGGACAAGAACCC-3′) was mapped to exon 13 of the human *CaMKK β* gene, whereas the antisense primers R1 (5′-TCTCATAAGGACACAAAGCC-3′) and R2 (5′-TCTCACAAGAGCACTTCCTC-3′) were complementary to sequences of exons 17 and 18, respectively. The PCR products were separated by agarose gel electrophoresis and examined under UV after ethidium bromide staining.

Autophosphorylation and Kinase Activity of Human CaMKK β Proteins—The pGEX-CaMK I plasmid encoding the human CaMK I fusion protein was kindly provided by Dr. Anthony R. Means (Duke University Medical Center, Durham, NC). The pGEX-CaMK IV plasmid was constructed by PCR amplification of the coding region of the human *CaMK*

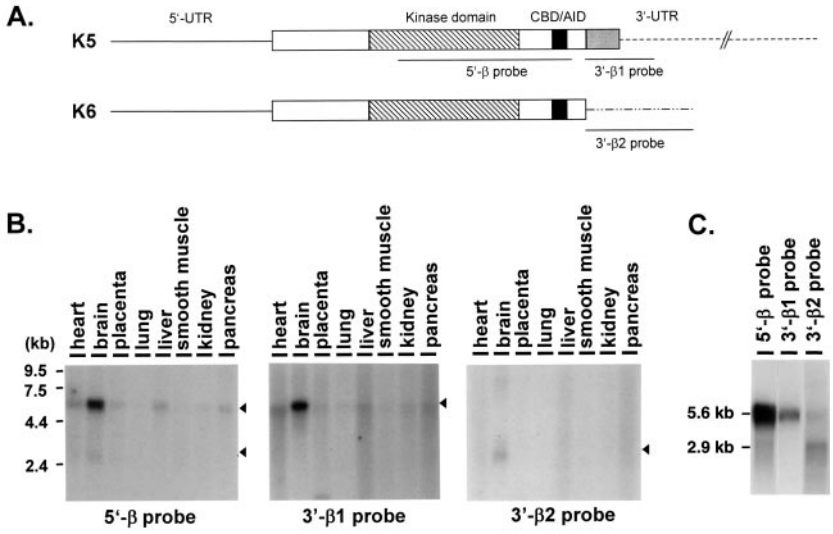

FIG. 1. **Northern blot analysis of human *CaMKK β* mRNA expression.** *A*, schematic representation of the two human *CaMKK β* cDNA clones, K5 and K6. The *boxed* regions represent the deduced open reading frames. The kinase domain and the overlapping Ca^{+2}/calmodulin binding domain (*CBD*) and autoinhibitory domain (*AID*) are indicated by the *hatched* and *black boxes*, respectively. The *stippled* region and 3′- untranslated region (*UTR*) in the K5 cDNA are encoded by sequences that differ from those of the K6 cDNA. The regions used for the generation of probes for Northern hybridization are indicated. The 5′-*β* probe was derived from a cDNA fragment corresponding to nt 1482–2367 of the K5 cDNA (*CaMKK β*1, GenBank™ accession number AF287630) and K6 cDNA (*CaMKK β*2, GenBank™ accession number AF287631). The 3′-*β*1 probe was derived from a K5 cDNA fragment from nt 2422–2774, and the 3′-*β*2 probe was derived from a K6 cDNA fragment from nt 2422–2960. *B*, human multiple tissue Northern blot analysis. *C*, Northern blot analysis of human glioblastoma/astrocytoma U-87 MG mRNA. A human multiple tissue RNA blot (H1 from CLONTECH) and mRNA prepared from human U-87 MG cells were analyzed and hybridized with probes derived from the 5′-region common to K5 and K6 cDNAs or the sequences specific to each cDNA. The 5.6 and 2.9 kb signals are indicated.

IV gene from brain cDNA and subcloned into pGEX-KG in the way that CaMK IV was fused in-frame with GST. The GST-CaMK I and GST-CaMK IV were expressed in *Escherichia coli* XA90 cells and affinity-purified as described (28). To express human CaMKK *β* isoforms, the cDNA encoding each isoform was amplified from the brain cDNA or corresponding EST clones by PCR and subcloned into the pFLAG-CMV2 vector (Eastman Kodak Co.). The identity of the resultant plasmids was confirmed by sequence analyses. Plasmids of pFLAG-CMV-CaMKK *β* and pFLAG-CMV were transfected into human non-small cell lung cancer cell line H-1299 by electroporation as described (30). Forty-eight hours post-transfection, cell lysates were prepared by incubating cells for 10 min on ice in lysis buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium orthovanadate, leupeptin (1 *μ*g/ml), and 1 mM phenylmethylsulfonyl fluoride. To immunoprecipitate FLAG-tagged CaMKK *β* protein, cell lysates (300 *μ*g protein) were preincubated with 1 *μ*g of mouse IgG at 4 °C for 30 min followed by the addition of 7 *μ*l of protein G-agarose beads (Life Technologies, Inc.) and incubated for an additional 30 min. After centrifugation at 13,000 × *g* for 10 min, supernatants were incubated with 3 *μ*g of mouse anti-FLAG monoclonal antibody M2 (Eastman Kodak Co.) at 4 °C for 1 h, and the antigen-antibody complex was precipitated by a further incubation of the mixture with 10 *μ*l of protein G-agarose beads for 3 h. The immunoprecipitates were washed with lysis buffer twice, followed by two washes with kinase buffer (25 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, and 10 mM $MgCl_2$). For autophosphorylation assay, the precipitates were resuspended in 10 *μ*l of kinase buffer containing 0.1 mM ATP, 5 *μ*Ci of [γ-^{32}P]ATP in the presence of 2 mM $CaCl_2$ plus 10 *μ*M CaM. For reactions carried out in the absence of CaM, 2 mM EGTA was added instead of 2 mM $CaCl_2$ and 10 *μ*M CaM. For kinase activity assay, 4 *μ*g of GST-CaMK I or 1 *μ*g of GST-CaMK IV was added to kinase buffer. The reaction was incubated at 30 °C for 20 min and terminated by boiling in Laemmli SDS-polyacrylamide gel sample electrophoresis buffer followed by electrophoresis on a 10% SDS-polyacrylamide gel. Phosphorylation was examined by autoradiography of the dried gel.

Chromosomal Localization of Human CaMKK β by Fluorescence in Situ Hybridization—BAC DNA was labeled with biotin-14-dATP with the BioNick™ DNA labeling system (Life Technologies, Inc.) according to the manufacturer's instructions. The human metaphase slide (Vysis Inc., Downers Grove, IL) was denatured in 70% formamide and 2× SSC at 76 °C for 5 min, dehydrated sequentially in 70, 80, and 90% ethanol, and air-dried. The labeled probe (200 ng) was mixed with 10 *μ*g of Cot-1 DNA and precipitated by ethanol. After centrifugation, the probe was re-dissolved in 10 *μ*l of hybridization buffer (70% formamide and 2.8× SSC) and loaded on the slide. After hybridization at 37 °C overnight in a humid chamber, the slide was washed 3 times with 50% formamide and 2× SSC at 45 °C for 10 min each, 1 time with 2× SSC at room temp for 5 min, and 3 times with PN buffer (0.1 M sodium phosphate buffer, pH 8.0, 0.1% Nonidet P40) at room temp for 10 min each. After blocking in PN buffer containing 5% nonfat dry milk at room temp for 1 h, the slide was incubated with 100 *μ*l of a fluorescein isothiocyanate-avidin (obtained from Vector Laboratories Inc., Burlingame, CA) solution (0.6 *μ*g of fluorescein isothiocyanate-avidin in blocking buffer) at 37 °C for 5 min. After washing twice with PN buffer at 37 °C for 10 min each, the slide was blocked in blocking buffer for 30 min and then incubated with 100 *μ*l of biotin-anti-avidin antibody (Vector) solution (1.2 *μ*g biotinylated-anti-avidin antibody in blocking buffer) at 37 °C for 5 min. After washing with PN buffer followed by another blocking procedure, the slide was incubated with fluorescein isothiocyanate-avidin as described above. After washing with PN buffer, the slide was counterstained with 4′,6-diamidino-2-phenylindole.

RESULTS

Two Major Species of Human CaMKK β Transcripts—We previously reported the isolation of two human *CaMKK β* cDNA fragments, termed K5 and K6, that have different 3′-sequences (28). To further delineate the expression of these two cDNAs, Northern blot analyses were performed using probes derived from the common 5′ region and the 3′-sequences specific to the individual cDNA species (Fig. 1*A*). A signal of 5.6 kb was readily detected when a human multiple tissue blot was hybridized against the probe derived from the 5′-sequences common to both *CaMKK β* cDNAs (Fig. 1*B*). The 5.6-kb mRNA species was highly expressed in brain and to a lesser degree in other tissues. We also noted a weak signal corresponding to 2.9 kb in the brain tissue. When the same blot was differentially hybridized with K5- or K6-specific probes, the 5.6-kb species was detected only by the K5-specific probe; on the contrary, the 2.9-kb species was detected only by the K6-specific probe. Similar results were observed when Northern analysis was performed with RNA prepared from human glioblastoma/astrocytoma U-87 MG cells (Fig. 1*C*). These results indicated that the two human *CaMKK β* cDNAs previously identified were derived from two distinct transcripts. Both transcripts are ex-

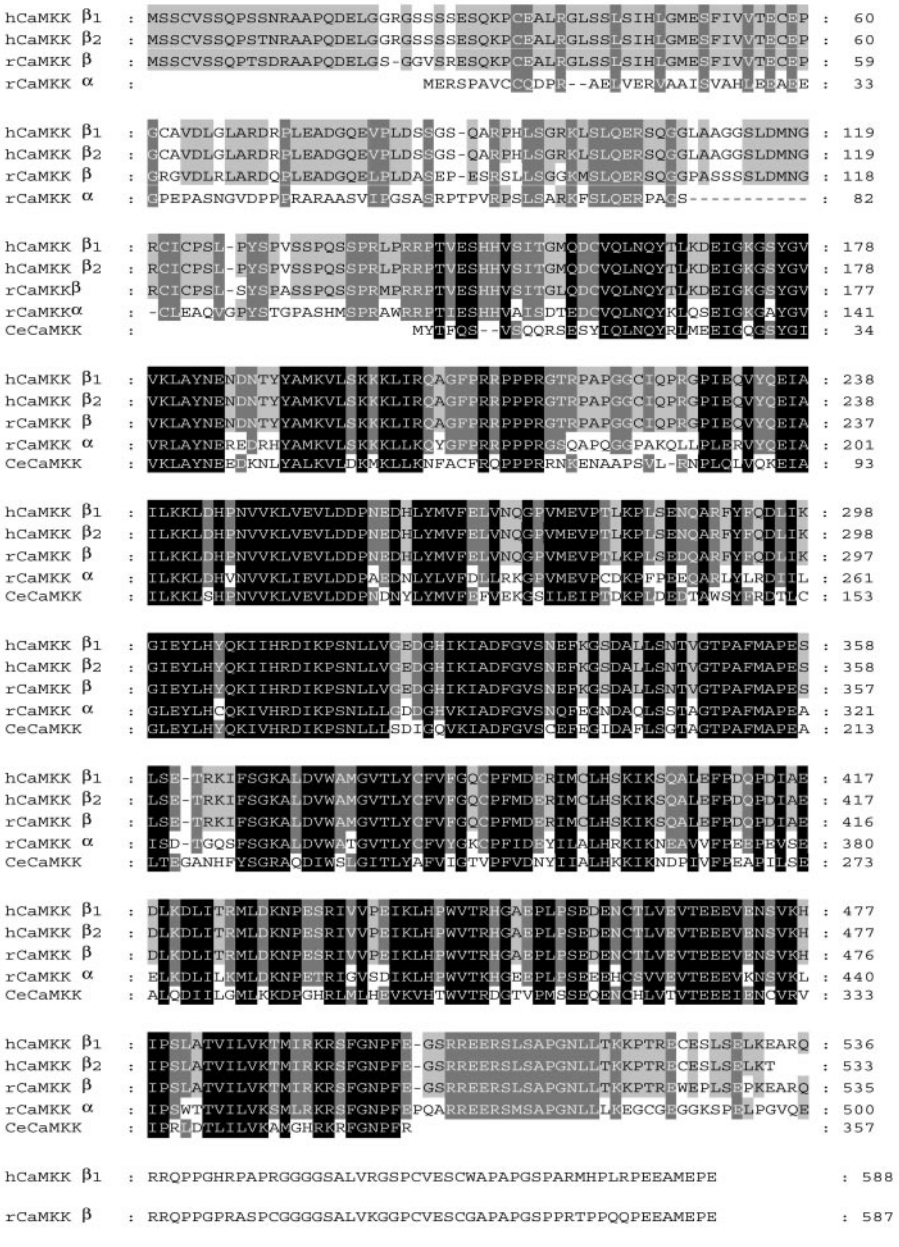

FIG. 2. **Amino acid sequence alignment of the CaMKK family.** Amino acid sequences of human (*h*) CaMKK β1 and β2, rat (*r*) CaMKK α, rat CaMKK β, and *C. elegans* (Ce) CaMKK are aligned. Amino acids are indicated in single-letter code. The residues that are identical or share similarity among five, four, and three CaMKK proteins are shaded in *black*, *dark gray*, and *light gray*, respectively. Gaps are introduced and shown by *dashed lines*.

pressed predominantly in the brain, with the 5.6-kb transcript as the major species. Full-length sequences of these two cDNAs were obtained through a combination of approaches. Rapid amplification of cDNA ends experiments were carried out to further extend the sequences of the 5′-end. A BAC clone containing the human *CaMKK* β gene was screened and analyzed for exonic regions encoding the 3′-ends. The human expressed sequence tag (EST) database was also searched for entries that match the sequence of human *CaMKK* β. The major transcript (5592 bp), designated as *CaMKK* β1, encodes an open reading frame of 588 amino acids that are identical to the recently published human CaMKK β sequence (15). The minor transcript (2960 bp) encodes the CaMKK β2, which contains 533 amino acids. The CaMKK β1 and β2 share an identical 532 amino acids at the N termini. The kinase catalytic domain and CaM binding domain are located at residues 165–419 and 475–500, respectively. A Pro/Arg-rich region was also identified in the catalytic region at residues 204–225. This region has been suggested to be involved in the recognition of CaMKK with CaMK I/CaMK IV (31). Fig. 2 shows the amino acid alignment of the human, rat, and *C. elegans* CaMKK isoforms. The human CaMKK β1 shares 97, 65, and 56% amino acid sequence identity to the rat CaMKK β, rat CaMKK α (19), and *C. elegans* CaMKK (31), respectively.

Genomic Structure of Human CaMKK β—To establish the molecular basis for expression of β1 and β2 transcripts, the genomic structure of *CaMKK* β was determined. λ phage clones (G5-1, G2-1, and G3-1) and a bacterial artificial chromosome (BAC number 2283L16) clone that contains sequences corresponding to the human *CaMKK* β gene were obtained. Sequence analysis of these genomic clones was performed using oligonucleotide primers derived from the cDNA sequences. The intron-exon boundaries were mapped by sequencing each exon in its entirety along with portions of the adjacent introns (Table I). The approximate size of each intron was estimated by PCR amplification of human genomic DNA using oligonucleotide primers flanking each intron. The human *CaMKK* β gene spans more than 40 kb and is organized into 18 exons. A graphic representation of the *CaMKK* β gene is shown in Fig. 3. All the exons are flanked by the canonical consensus splice sites, AG at the 3′ splice site and GT at the 5′ splice site (Table I). The exons range in size from 22 nucleotides (nt) (exon 8) to 3174 nt for the

TABLE I

Exon-intron junction sequences of the human CaMKK β gene

The exon sequence is represented by uppercase letters, and the intron sequence is represented by lowercase letters. The sequences of exon-intron boundaries were determined by comparing the sequence of genomic DNA and the cDNA sequence of the human *CaMKK β* gene. The canonical consensus splice sites; ag at the 3′ splice site and gt at the 5′ splice site, are in boldface. The GenBank™ accession numbers for individual exons are AF321389 (exon 1), AF321390 (exon 2), AF321575 (exon 3), AF321391 (exon 4), AF321392 (exon 5), AF321393 (exon 6), AF321576 (exon 7), AF321577 (exon 8), AF321394 (exon 9), AF321395 (exon 10), AF321396 (exon 11), AF321397 (exon 12), AF321398 (exon 13), AF321399 (exon 14), AF321400 (exon 15), AF321578 (exon 16), AF321401 (exon 17), and AF321402 (exon 18).

Splice acceptor sequence	Exon	Exon size	Splice donor sequence	Intron size
		bp		*kb*
	1	764	AGCAAA**gt**aagtgcca	~6.0
ctttctgc**ag**GTGGCT	2	530	ATGCAG**gt**gcgtgagc	~3.0
tactctct**ag**GACTGT	3	48	GGAAAG**gt**aaatatcc	~1.2
ctttatcc**ag**GGCTCC	4	54	TACTAT**gt**gagtatcc	~1.0
tttcctgc**ag**GCAATG	5	52	TTCCAC**gt**gagtttgg	~3.0
tttccttc**ag**GTCGCC	6	134	GTGGAG**gt**aaaagggt	~4.0
gctgtgca**ag**GTCCCTG	7	37	ACATGG**gt**aaggaagc	~4.9
ttccctac**ag**TGTTCG	8	22	CCAAGG**gt**gagtgtcc	~0.1
gtcctttc**ag**GCCCGT	9	89	AGTACT**gt**gagcagca	~1.9
gcctcccc**ag**TACACT	10	200	GGGAAG**gt**aagcttgg	~0.6
tttcttgc**ag**GCCTTG	11	54	GGCCAG**gt**aagagggc	~3.0
ctctttgc**ag**TGCCCA	12	74	AGACCA**gt**gagtatct	~0.3
ccatcccc**ag**GCCCGA	13	88	ATCAAG**gt**acccttgg	~1.2
ttcgttga**ag**CTGCAC	14	129	ACCGTG**gt**aaggtgga	~3.0
ccctcttc**ag**ATCCTG	15	101	GCTCAC**gt**gagggccg	~0.6
gggattcc**ag**CAAAAA	16	43	CTCAAG**gt**aacacccg	~0.8
atgtgttt**ag**ACCTAG	17	541 (β2)	TACACTacgtggcctt	~3.0
cggtgagc**ag**GAAGC	18	3174 (β1)	AATGGTgccaagcctg	

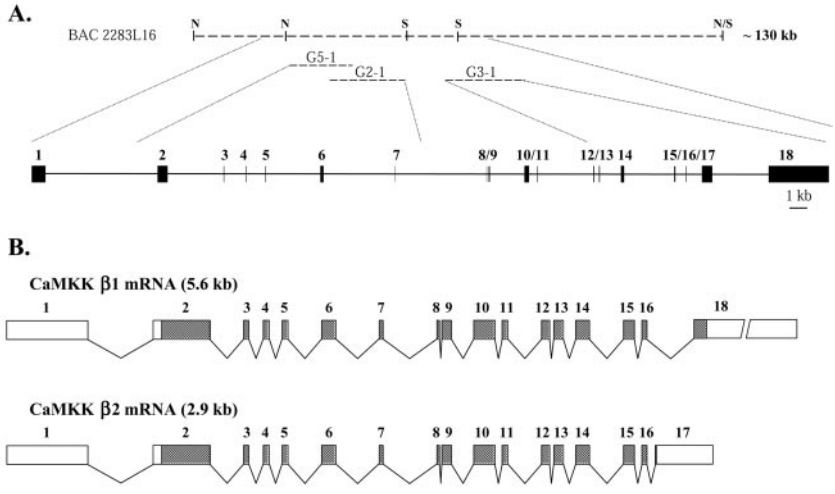

FIG. 3. **Genomic structure and transcripts of human *CaMKK β*.** *A*, the human *CaMKK β* locus. Exons and introns are drawn schematically and to scale. Exons are indicated by *solid boxes* and *numbered* above, whereas introns are indicated by the *horizontal line* between exons. The *dashed lines* on top represent the relative size and position of the *CaMKK β* genomic clones, the BAC clone 2283L16 and the λ phage clones G5-1, G2-1, and G3-1. The restriction endonucleases that cut the BAC clone at the positions indicated are *Not*I (*N*) and *Sal*I (*S*). *B*, exons encoding *CaMKK β*1 (5.6 kb) and *β*2 (2.9 kb) transcripts. Exons are shown in *boxes* and *numbered* above. The *hatched boxes* represent translated regions, and the *empty boxes* represent untranslated regions.

last exon. The introns range in size from 143 nt (intron 8) to 6 kb (intron 1). Exon 1 is non-coding. The translation initiation codon ATG is located in the second exon, which is separated from the first exon by the large intron 1. An in-frame termination codon is located 276 bp upstream of the ATG codon in the coding sequence. The conserved kinase catalytic domain spans exons 3–13; the consensus ATP binding motif G*X*G*XX*G*X*V is encoded by exons 3 and 4, and the activation loop is encoded by exon 10. Sequences encoding the calmodulin-binding site and autoinhibitory domain encompass exons 14 and 15. The *β*1 and *β*2 transcripts are encoded by the *CaMKK β* gene through differential usage of the polyadenylation sites located in the last and penultimate exons. Both the *β*1 and *β*2 transcripts contain sequences derived from exons 1–16, and the *β*1 transcript utilizes exon 18, whereas the *β*2 transcript utilizes exon 17, to conclude their 3′-termini (Fig. 3*B*). Exon 18 contains sequences encoding the C-terminal 56 amino acids of the CaMKK *β*1 followed by a 3.0-kb 3′-untranslated region. Exon 17 introduces only one amino acid and a stop codon to the open reading frame of the CaMKK *β*2 followed by a 535-bp long 3′-untranslated region. The polyadenylation signals were identified for *CaMKK β*1 and *β*2 at exon 18 (AATAAA) and exon 17 (TATAAA), respectively.

Transcriptional Initiation Site(s) of Human CaMKK β—To determine the human *CaMKK β* transcription initiation site(s), primer extension was performed using mRNA derived from human U-87 MG and H-1299 cell lines as templates and a reverse primer designed from the sequence of the first exon (Fig. 4*A*). A major extension product of 364 bp was identified with template derived from U-87 MG cells but not with that of H-1299 cells. Several weak extension products were also observed. These results suggest that the transcription start site of human *CaMKK β* mRNA is 823 bp from the ATG codon and located within the sequences that match the 5′-YC(A/T)GYYYY-3′ (Y, pyrimidine) consensus initiator sequence (Fig. 4*B*). Sequence analysis of the 5′-flanking region of the human *CaMKK β* gene revealed that it lacks the canonical TATA box or CAAT box (Fig. 4*B*). However, the consensus binding sequences for several transcription factors including p300, LyF-1, AML-1, and GATA-1 were identified.

Identification of Alternatively Spliced CaMKK β Transcripts by RT-PCR Analysis—We previously showed that a *CaMKK β* K6 cDNA variant contained an in-frame deletion of a stretch of 129 nucleotides near its 3′-end (28). Alignment of the cDNA sequence to the genomic sequences revealed that the variant resulted from alternative RNA splicing of exon 14. RT-PCR was

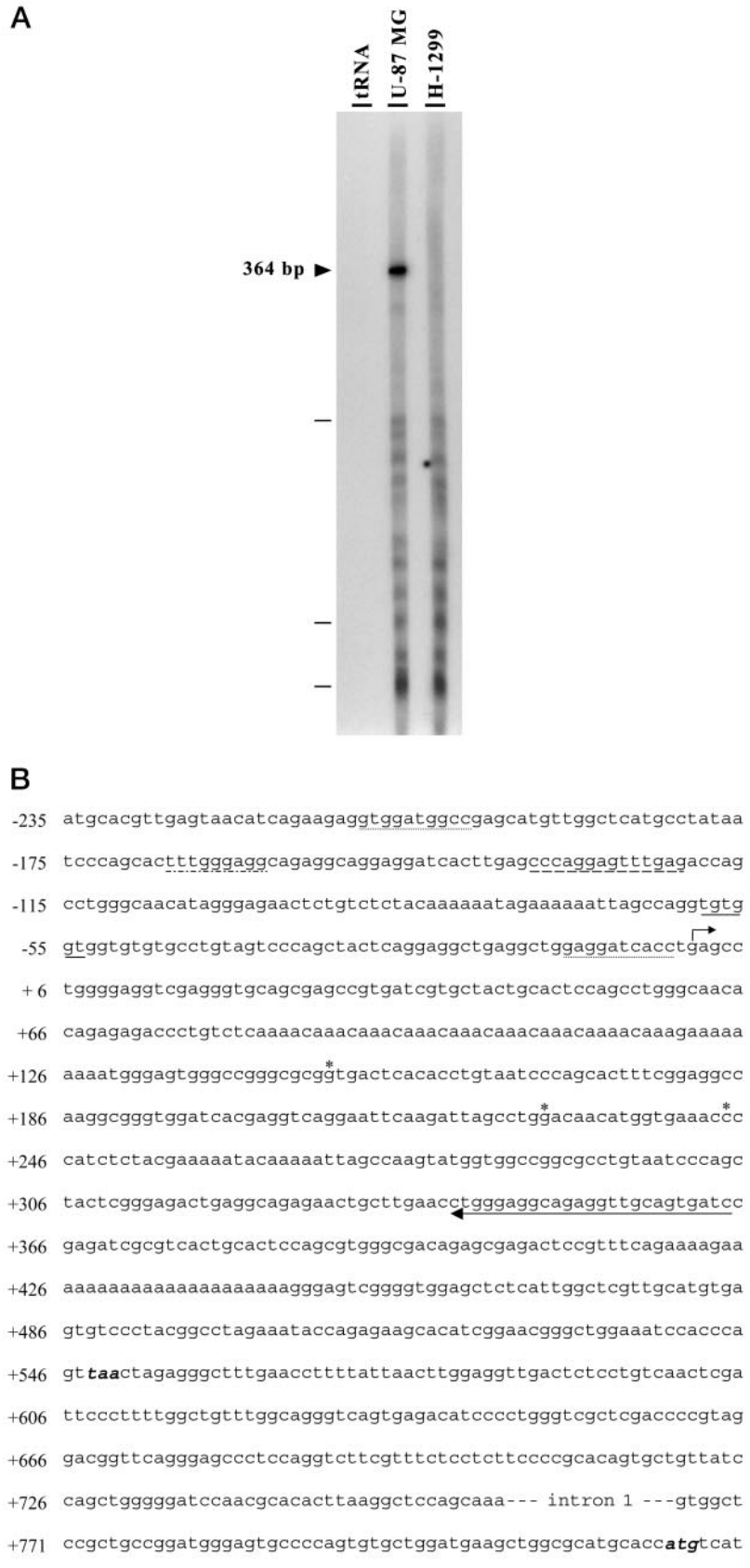

FIG. 4. **Primer extension analysis and the sequences of the promoter and 5′-untranslated region of human *CaMKK β* gene.** *A*, determination of transcription initiation site by primer extension analyses. Messenger RNA prepared from human U-87 MG and H-1299 cells and yeast tRNA were annealed to the end-labeled oligonucleotide and reverse-transcribed as described under "Experimental Procedures." Primer extension products were analyzed on a denaturing, polyacylamide gel together with a sequencing ladder. The *arrow* indicates the major primer extension product of 364 bp. Minor products of 121, 137, and 216 bp are indicated by *bars*. *B*, the promoter and 5′-untranslated sequences of the human *CaMKK β* gene. The promoter and 5′-untranslated sequences of the human *CaMKK β* gene were obtained by sequencing analysis of BAC clone 2283L16 and *CaMKK β* cDNA. The location of the reverse primer for primer extension analysis is indicated by the *underline arrow*. Transcription initiation site 823 bp upstream of the ATG codon is numbered as +1 in the nucleotide sequence and is indicated by a *bent arrow*. The sites corresponding to the start of the minor primer extension products are indicated by *asterisks*. Putative binding sites for p300 and GATA-1 are *underlined* with a *dashed line* and *dotted lines*, respectively. The AML-1 and Lyf-1-binding sites are *underlined* with a *solid line* and a *dashed* and *dotted line*, respectively. The ATG codon and the upstream in-frame stop codon TAA are in *italic boldface*.

performed to explore the presence of this and additional alternatively spliced *CaMKK β* transcripts in various human tissues and cell lines (Fig. 5). To detect the transcripts with different 3′-terminal sequences, oligonucleotide F1 located in exon 13 was used as forward primer, and oligonucleotides R1 complementary to the sequences in exon 18 and R2 complementary to the sequences in exon 17 were used as reverse primers. As shown in Fig. 5*A*, six transcripts (four β1-type and two β2-type) were amplified upon RT-PCR analysis using RNA templates prepared from human placenta (*lane 1*), human

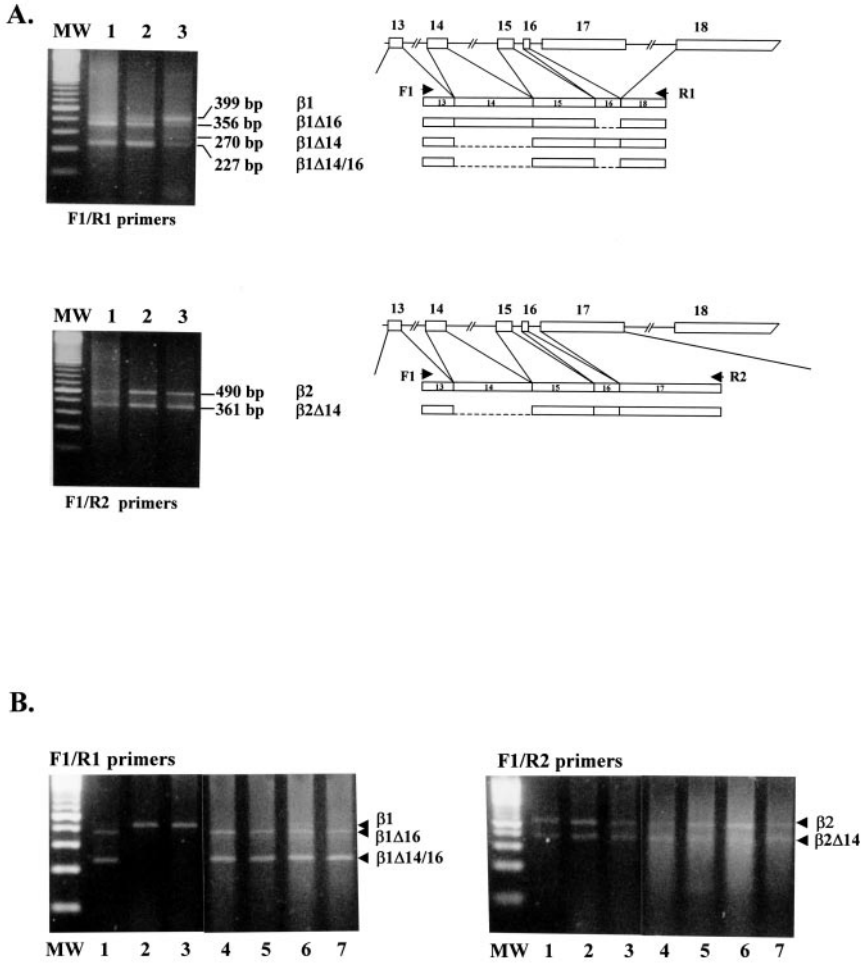

FIG. 5. **RT-PCR analyses of alternatively spliced *CaMKK β* transcripts.** *A*, schematic representation and RT-PCR analyses of human *CaMKK β* isoforms. Five μg of total RNA prepared from human placenta (*lane 1*), U-87 MG cells (*lane 2*), and U-138 MG cells (*lane 3*) were used in first-strand cDNA synthesis and subjected to PCR. Oligonucleotide F1 located in exon 13 was used as the sense primer, and R1 mapped to exon 18 and R2 mapped to exon 17 were used as the reverse primers to amplify β1- and β2-related transcripts. PCR products were separated by 4% agarose gel and examined under UV light after ethidium bromide staining. To the right, schematic representations of the exonic sequences encoding the individual isoforms are shown. The genomic structure of the *CaMKK β* gene from exon 13 to exon 18 is shown on top of each drawing; exons are shown in *boxes* and *numbered* above, and introns are indicated by *horizontal lines* between exons. Corresponding exonic sequences amplified by RT-PCR are shown under the genomic structure. The coding exons are indicated by *boxes*, and the skipped exons are indicated by *broken lines*. *MW*, molecular mass. *B*, expression of *CaMKK β* transcripts in human brain tissues by RT-PCR analyses. RNA was prepared from human placenta (*lane 1*), normal brain tissues (*lanes 2* and *3*), and brain tumor tissues (*lanes 4–7*) and subjected to RT-PCR analyses using F1/R1 and F1/R2 as primers. Various CaMKK β transcripts were amplified as indicated.

brain tumor-derived cell lines U-87 MG (*lane 2*), and U-138 MG (*lane 3*). These PCR products were individually purified from the gel and subcloned into pGEM-T. Sequence analysis of the β1-related transcripts revealed that one corresponded to the full-length β1 sequence encoded by exons 13–16 plus exon 18, and the others were alternatively spliced variants in which the internal exons 14 or 16 or both were skipped. Similarly, the two β2-related transcripts were identified to be the unspliced β2 transcript encoded by exons 13–17 and an alternatively spliced variant lacking exon 14. Alternative splicing of exon 14 resulted in an in-frame deletion of 43 amino acids, whereas deletion of exon 16 results in a change of the open reading frame that leads to a premature stop of translation. Most of these *CaMKK β* isoforms were also detected in the brain. Fig. 5*B* shows the results of RT-PCR analyses of RNA prepared from normal brain tissues (*lanes 2* and *3*) and brain tumor tissues (*lanes 4–7*). It was interesting to note that the unspliced full-length β1 transcript represented the predominantly expressed species in the normal brain tissues examined as compared with the alternatively spliced transcripts, whereas the spliced variants appeared to be more abundantly expressed in the brain tumor tissues. In contrast, the full-length β2 transcript and the alternatively spliced β2Δ14 variant were expressed at relatively comparable levels in the normal brain and brain tumor tissues.

Kinase Activity of Human CaMKK β Isoforms—It is well established that CaMK I and CaMK IV are phosphorylated and activated by CaMKK. To determine whether the CaMKK isoforms generated through alternative RNA processing exhibit similar kinase activity to phosphorylate downstream substrates, *in vitro* kinase assay was performed. The human CaMKK β isoforms were overexpressed in human non-small cell lung cancer H-1299 cells as FLAG-CaMKK fusion proteins. The fusion proteins were immunoprecipitated with monoclonal antibody recognizing the FLAG tag, and the immunoprecipitates were subjected to kinase assay utilizing affinity-purified GST-CaMK I and GST-CaMK IV as substrates. As shown in Fig. 6*A*, both CaMKK β1 and CaMKK β2 strongly phosphorylate GST-CaMK I in the presence of Ca^{2+}/CaM. The CaMKK β1Δ16 variant retained its kinase activity, whereas phosphorylation of GST-CaMK I by CaMKK βΔ14 or CaMKK βΔ14/16 was hardly detectable. Autophosphorylation of CaMK I was observed in the presence of Ca^{2+}/CaM as shown in the mock-transfected sample (*lane 2*). In parallel experiments, the phosphorylation of GST-CaMK IV by CaMKK β was examined (Fig. 6*B*). Similar results were obtained, *i.e.* GST-CaMK IV was phosphorylated by CaMKK β1 or CaMKK β2 in a Ca^{2+}/CaM-dependent manner; deletion of exon 16 did not affect kinase activity, whereas deletion of exon 14 abolished kinase activity. These results showed that CaMKK β isoforms β1, β2, and β1Δ16, although they possess divergent C termini, exhibit similar activity toward phosphorylating the downstream substrates, CaMK I and CaMK IV. In contrast, an in-frame deletion of the internal exon 14 significantly impaired kinase activity.

Autophosphorylation of Human CaMKK β Isoforms—We next examined the autophosphorylation of CaMKK β isoforms using immunoprecipitated FLAG-CaMKK β fusion proteins that were overexpressed in H-1299 cells. As shown in Fig. 7, CaMKK β1 and CaMKK β2 were capable of autophosphorylating themselves. Robust enhancement of autophosphorylation was observed in the presence of Ca^{2+}/CaM. Consistent with the kinase activity of the alternatively spliced CaMKK β variants,

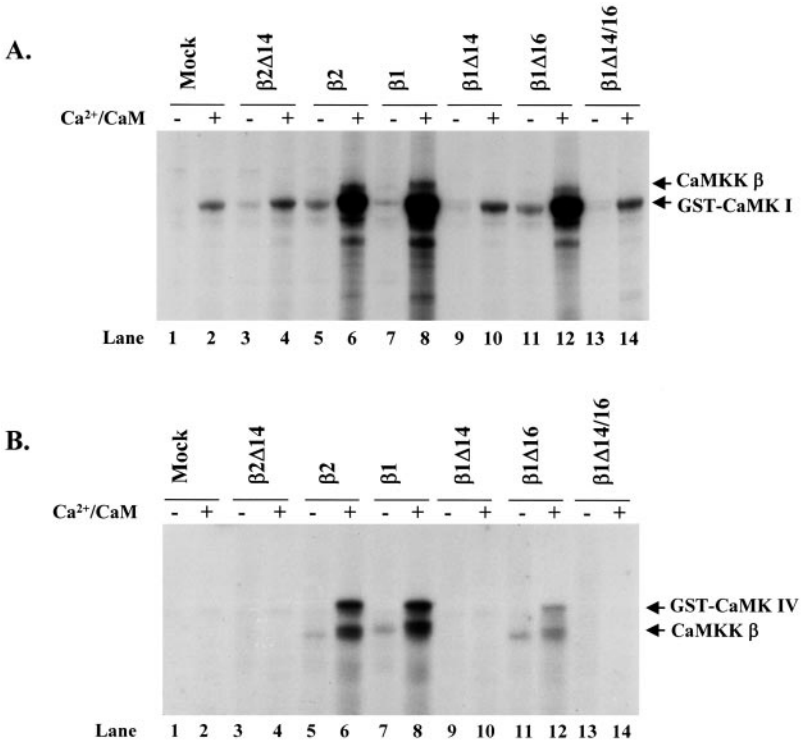

FIG. 6. **Kinase activity of human CaMKK β isoforms.** *A*, phosphorylation of GST-CaMK I. *B*, phosphorylation of GST-CaMK IV. pFLAG-CMV and the same plasmid expressing individual CaMKK β isoforms were transfected into human non-small cell lung cancer H-1299 cells, respectively. The CaMKK β fusion proteins were shown to be expressed at comparable levels in H-1299 cells. The expressed FLAG-CaMKK β fusion protein was immunoprecipitated with anti-FLAG monoclonal antibody M2 and subjected to kinase assay using bacterially expressed and affinity-purified GST-CaMK I and GST-CaMK IV fusion proteins as substrates. For kinase assay, the immunoprecipitated FLAG-CaMKK β fusion protein was resuspended in 20 μl of kinase buffer containing 0.1 mM ATP, 5 μCi of [γ-^{32}P]ATP, 4 μg of GST-CaMK I, or 1 μg of GST-CaMK IV in the presence of 2 mM $CaCl_2$ plus 10 μM CaM (*lanes 2*, *4*, *6*, *8*, *10*, *12*, and *14*). For reactions carried out in the absence of CaM, 2 mM EGTA was added instead of Ca^{2+}/CaM (*lanes 1*, *3*, *5*, *7*, *9*, *11*, and *13*). Reactions were incubated at 37 °C for 20 min, and the reaction mixtures were separated on 9% SDS-polyacrylamide gel and exposed to x-ray film after drying the gel. Plasmids used for transfection included pFLAG-CMV (*Mock*, *lanes 1* and *2*), pFLAG-CMV-CaMKK β2Δexon 14 (β2Δ14, *lanes 3* and *4*), pFLAG-CMV-CaMKK β2 (β2, *lanes 5* and *6*), pFLAG-CMV-CaMKK β1 (β1, *lanes 7* and *8*), pFLAG-CMV-CaMKK β1Δ exon14 (β1Δ14, *lanes 9* and *10*), pFLAG-CMV-CaMKK β1Δexon 16 (β1Δ16, *lanes 11* and *12*), pFLAG-CMV-CaMKK β1Δexons 14 and 16 (β1Δ14/16, *lanes 13* and *14*).

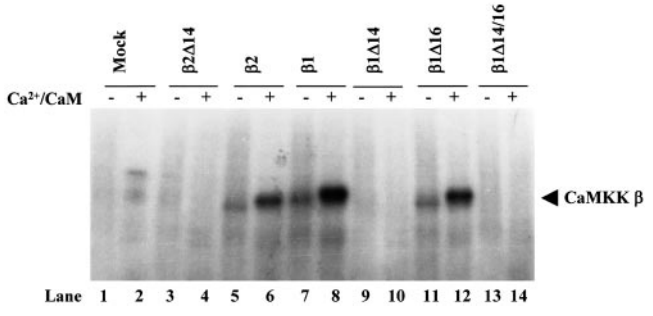

FIG. 7. **Autophosphorylation of human CaMKK β isoforms.** The CaMKK β isoforms were overexpressed in human H-1299 cells as FLAG-CaMKK β fusion proteins and immunoprecipitated as described in the legend of Fig. 6. For autophosphorylation assay, the immunoprecipitates were resuspended in 20 μl of kinase buffer containing 0.1 mM ATP, 5 μCi of [γ-^{32}P]ATP in the presence of 2 mM $CaCl_2$ plus 10 μM calmodulin (*lanes 2*, *4*, *6*, *8*, *10*, *12*, and *14*). For reactions carried out in the absence of CaM, 2 mM EGTA was added instead of Ca^{2+}/CaM (*lanes 1*, *3*, *5*, *7*, *9*, *11*, and *13*). Reactions were incubated at 37 °C for 20 min, and the reaction mixtures were separated by 9% SDS-polyacrylamide gel and exposed to x-ray film after drying the gel.

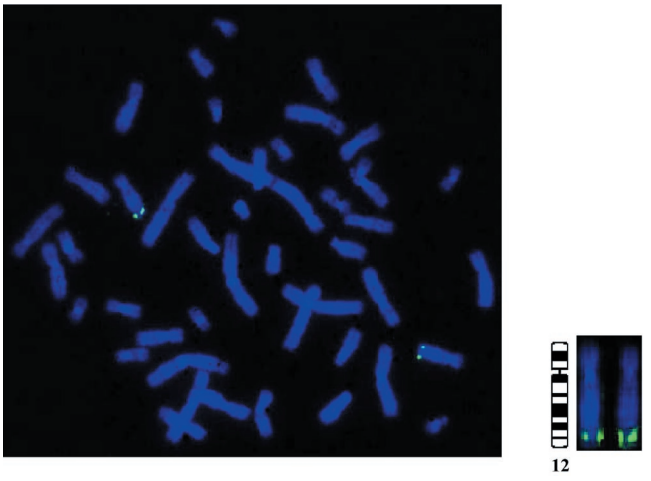

FIG. 8. **Chromosomal localization of human *CaMKK β* locus by fluorescence *in situ* hybridization analysis.** BAC clone 2283L16, which contains the *CaMKK β* gene, was biotin-labeled and hybridized to human metaphase slides followed by counterstaining with 4′,6-diamidino-2-phenylindole. Two pairs of fluorescent signals were observed, indicating the hybridization of the probe to the two pairs of sister chromatids. The human *CaMKK β* locus was assigned to the chromosome 12q24.2 sub-region.

alternative splicing of exon 14 gave rise to CaMKK β proteins incapable of undergoing autophosphorylation, whereas splicing of exon 16 did not affect autophosphorylation activity.

Chromosomal Localization of Human CaMKK β Locus—We previously showed that the human *CaMKK β* gene is located on chromosome 12 by PCR analysis of a human/rodent somatic cell hybrid mapping panel (28). To further map the *CaMKK β* locus, fluorescence *in situ* hybridization analysis was performed utilizing biotin-labeled BAC clone as probe. Compared with the 4′,6-diamidino-2-phenylindole banding pattern, fluorescent signals of the *CaMKKβ* gene were assigned to chromosome 12q24.2 (Fig. 8).

DISCUSSION

The human *CaMKK β* gene spans a minimum of 40 kb and comprises 18 exons. Multiple transcripts are generated from the human *CaMKK β* gene through alternative RNA processing. Two major types of transcripts are produced by differential usage of polyadenylation sites located in the last and penultimate exons. Both transcripts contain sequences encoded by exons 1–16 but differ 3′ of this common region. The predominant β1 transcript (5.6 kb in size) skips over exon 17 and splices exon 16 to exon 18 where it polyadenylates. The minor species, β2 (2.9 kb), is produced by inclusion of exon 17, where it concludes its C terminus. Additional forms of transcripts are generated through alternative splicing of the internal exons 14 and/or 16.

Human CaMKK β1 shares 97% amino acid sequence homology to rat CaMKK β. Like rat CaMKK β, the human enzyme is ubiquitously expressed, with the brain as the site showing highest expression (28). In rat brain, CaMKK β displayed an expression pattern distinct from the CaMKK α (15, 18). CaMKK α-immunoreactivity was distributed in neurons throughout the brain, except in the cerebellar cortex. CaMKK β-immunoreactivity was relatively restricted in some neuronal populations. The highest level of CaMKK β was observed in the cerebellar granule cell layer, and moderate immunoreactivity was observed in the cerebral cortex, hippocampal formation, caudate putamen, pontine nuclei, cochlear nucleus, and molecular layer of the cerebellum (15, 18). In the present study, we further examined the regional expression of human *CaMKK β* in the brain by dot blot analysis using human RNA Master Blot (CLONTECH) to which poly(A)$^+$ RNAs from different regions of the brain were immobilized in separate dots. Our results showed that human *CaMKK β* was highly expressed in the cerebellum, moderately expressed in the occipital lobe, putamen, subthalamic nucleus, caudate nucleus, frontal lobe, and cerebral cortex, and weakly expressed in the amygdala, hippocampus, medulla oblongata, thalamus, and substantis nigra (data not shown). It appears that the human and rat *CaMKK β* orthologs encode proteins that are not only structurally similar but also share similar expression patterns. To examine the expression patterns of human *CaMKK* β1 and β2 transcripts, the human RNA Master Blot was hybridized against β1- or β2-specific probes derived from the unique 3′-terminal sequences of the transcripts. Similar expression patterns were found for both transcripts.

Many genes have been described and characterized that use alternative polyadenylation sites at the 3′-end of their mRNAs according to their cellular environment (32). By a skipped exon mechanism, there are genes that encode two or more mRNAs by using the first alternative 3′-terminal exon with its poly(A) site (pA1) or by skipping that exon entirely and splicing the second 3′-terminal exon into the transcript using pA2 instead (32). By selecting alternative polyadenylation sites, the calcitonin/CGRP (calcitonin gene-related peptide) gene generates transcripts encoding predominantly calcitonin in thyroid C cells or CGRP in the nervous system (33, 34). Studies of mice with a calcitonin/CGRP transgene showed tissue-specific differences in calcitonin/CGRP expression, suggesting that a specific regulatory mechanism restricted primarily to neurons is required for CGRP expression (35). More recently, the human CUTL1 gene (Cut (*Drosophila*)-like 1) was shown to give rise to the CDP/Cut (CCAAT displacement protein/human Cut) and CASP (Cut alternatively spliced product) transcripts (36). Both transcripts contain exons 2–14; exon 14 is spliced to exon 15 to generate CDP/Cut transcripts, which contain exons 15–24 or to exon 25 to produce CASP transcript containing exons 25–33 (36). The production of CDP/Cut or CASP mRNA was suggested to depend on the competition between cleavage at the end of exon 24 and splicing between exon 14 and 25 (resulting in the skipping of exons 15–24) (36). It was noted that the polyadenylation signal AAUAAA at the end of exon 24 is embedded within the sequence AAAAUAAAA, and the presence of an excess of A residues may lead to inefficient processing of the primary transcripts (37). Therefore, the primary transcripts that are elongated up to exon 33 may invariably be spliced between exons 14 and 25, with the possible cleavage downstream of exon 24 (36). In the present study, the human *CaMKK β* gene was also processed through the skipped exon mechanism to generate β1 and β2 transcripts with different 3′-termini. The *CaMKK* β1 encodes 588 amino acids, whereas the *CaMKK* β2 encodes 533 amino acids. Both contain common exons from 1 to 16 that encode the first 532 amino acids. *CaMKK* β2 uses exon 17 as its 3′- untranslated region and poly(A) site, whereas *CaMKK* β1 skips that exon and splices exon 18 into its transcript where it polyadenylates. Consistent with our observation that *CaMKK* β1 is the predominant transcript, we found that *CaMKK* β1 utilizes the consensus polyadenylation signal AAUAAA located in exon 18, whereas *CaMKK β2* utilizes the atypical polyadenylation signal UAUAAA located in exon 17. Compared with the AAUAAA motif, the UAUAAA sequence represents a weaker signal for the recognition and binding by CPSF (cleavage and polyadenylation specificity factor) (32). We speculate that the majority of the primary *CaMKK β* transcripts are occupied by the CPSF in the AAUAAA site in exon 18 to generate β1 transcripts. The differential processing of primary transcripts from a number of genes through alternative poly(A) site choice has been shown to be a cell cycle-dependent, tissue-specific, or developmentally specific event (32). The regulated expression of these genes may be sensitive not only to the levels of general splicing and polyadenylation factors but also to gene-specific splicing factors that facilitate either the inclusive or the skip-over splice. The detailed mechanism underlying the production of human CaMKK β1 and β2 and the biological significance of this processing event require further study.

Human CaMKK β1 and β2 share identical N-terminal 532 amino acids but differ at their C termini. In the present study, additional forms of *CaMKK β* transcripts were also identified in human tissues and tumor-derived cell lines that were generated through alternative splicing of the internal exons 14 and/or 16. Skipping of exon 16 leads to a change of the open reading frame yielding a third C terminus that stops prematurely. Skipping of exon 14 leads to an in-frame deletion of 43 amino acid residues (amino acids 442–484) near the C terminus. Alternative splicing is a common mechanism that creates a variety of proteins with constant and variable functional domains from a single gene by RNA processing (38). Members of the CaMK family also contain various isoforms by means of alternative splicing (39, 40). The rat *CaMK* Iβ is differentially spliced into two isoforms (designated as β1 and β2) with distinct C termini (39, 41). These isoforms are developmentally regulated, with the β1 isoform present in rat embryos from day 18 and the β2 isoform present from day 5 postnatally. More than a dozen alternatively spliced *CaMK II* transcripts derived from four genes (α, β, γ, and δ) are differentially expressed in different human and rat tissues or cell lines (3, 42–44). By RT-PCR analyses, we demonstrated that *CaMKK* β1 represented the predominantly expressed species in normal brain tissues, whereas β1Δ14/16 and β1Δ16 were more abundantly expressed in brain tumor and placenta tissues. The distinct expression patterns of the unspliced and spliced *CaMKK β* variants were also observed in two human tumor-derived cell lines, U-87 MG and U-138 MG. In contrast, the relative abun-

dance of β2 and the alternatively spliced β2Δ14 variant remained unchanged in the tissues and cell lines examined. We also found that CaMKK β1 and β2 exhibit comparable kinase activities to phosphorylate downstream substrate kinases. Deletion of exon 16 did not affect kinase activity, whereas deletion of exon 14 yielded an inactive *CaMKK β* protein. It is poorly understood how the heterogeneity of the C termini of the *CaMKK β* isoforms affects its biological function. The C terminus of CaMKK II has been suggested to play a role in its subunit association (20, 21). Whether variant CaMKK β isoforms with different C termini would affect the protein association with itself or other proteins is not clear. Nevertheless, our findings warrant further study to dissect the mechanism that regulates the differential expression of CaMKK β isoforms and to determine the role of CaMKK β-mediated signaling pathways in different tissues under both physiological and pathophysiological conditions.

In the human expressed sequence tag (EST) database, we identified several entries derived from different tissues that contain sequences corresponding to the various *CaMKK β* transcripts described in this study (the *CaMKK β*1, Integrated Molecular Analysis of Genomes and their Expression clones 752659 and 824643; *CaMKK β*2, Integrated Molecular Analysis of Genomes and their Expression clones 2559582 and 2716667; *CaMKK β*1Δ14/16, Integrated Molecular Analysis of Genomes and their Expression clone 767832; *CaMKK β*1Δ16, Integrated Molecular Analysis of Genomes and their Expression clone 2117038). These findings further support our observations that the human *CaMKK β* gene is expressed in various isoforms through alternative splicing and polyadenylation. Consistent with our findings, Anderson *et al.* (15) detect two closely spaced immunoreactive bands in rat brain homogenate by Western blot analyses using antibodies raised against either the N (amino acid residues 28–49)- or C (amino acid residues 571–587)-terminal peptides of rat CaMKK β. Similarly, Sakagami *et al.* (18) also detect two immunoreactive bands at 70 and 73 kDa in rat brain homogenate by Western blot analysis using monoclonal antibody raised against rat CaMKK β peptide (amino acids 520–587) (18). In the latter report, the authors further noted that the closely spaced doublets migrated slightly faster than the full-length CaMKK β overexpressed in COS cells. It would be interesting to verify whether the two closely spaced doublets identified in rat brain homogenate represent alternatively spliced variants corresponding to the human CaMKK β isoforms from which exons 14 and/or 16 are removed. The result obtained from this study will also provide us information regarding whether the orthologous human and rat *CaMKK β* genes are conserved in genomic organization and are expressed through similar post-transcriptional RNA processing event.

Deletion of exon 14 rendered CaMKK β largely inactive upon phosphorylating its downstream targets, CaMK I and CaMK IV. This is likely a result of the interference of its interaction with calmodulin. In a previous study using site-directed mutagenesis and a synthetic peptide, Tokumitsu *et al.* (45) identify the region of the calmodulin binding site (residues 438–463) in rat CaMKK α. By NMR spectroscopic study, Osawa *et al.* (46) determine the structure of calcium-bound calmodulin (Ca^{+2}/CaM) complexed with the 26-residue peptide corresponding to the CaMKK α CaM-binding site. In this complex, the CaMKK α peptide was found to form a fold comprising an α helix (residue 444–454) and a hairpin-like loop (residue 455–459) whose C terminus folds back onto the helix. Both the α helix and the hairpin-like loop are involved in the interaction of CaMKK with CaM, and Trp^{444} and Phe^{459} were identified as the anchoring residues to the C- and N-terminal domain of CaM. Mutation of Phe^{459} to Asp completely abolished Ca^{+2}/CaM binding. By sequence alignment, the amino acids 475–500 of human CaMKK β correspond to the Ca^{+2}/CaM binding site, with Leu^{481} and Phe^{496} as the anchoring residues, respectively. Alternative splicing of exon 14 gives rise to a CaMKK β variant with an in-frame deletion of residues 442–484, which lacks the first 10 residues (including the C-terminal anchoring residue) of the Ca^{+2}/CaM binding site. This may impair Ca^{+2}/CaM binding and lead to inactivation of CaMKK β. In *Drosophila*, up to 18 different alternatively spliced CaMK II variants with heterogenoeous C termini covering the CaM binding domain were generated from a single gene (47). Seven variants were shown to have different binding affinity for CaM (47). These findings support that CaMK pathways may be regulated through alternative RNA splicing to generate isoforms that exhibit distinct kinase activity and calmodulin binding activity.

In summary, we have demonstrated that the human *CaMKK β* gene is organized into 18 exons and 17 introns and is localized to chromosome 12q24.2. Multiple transcripts are produced through alternative splicing and polyadenylation. These CaMKK β isoforms, except the ones in which exon 14 is deleted, undergo autophosphorylation in the presence and absence of Ca^{2+}/CaM, whereas the binding of Ca^{2+}/CaM is required for efficient phosphorylation of the downstream target kinases GST-CaMK I and GST-CaMK IV. The CaMKK β isoforms are differentially expressed in human tissues and cell lines. The diversity of human CaMKK β isoforms with heterogeneous C termini with distinct kinase activity and their relative abundance in different tissues further demonstrate the complexity of the regulation of the CaMKK-CaMK signaling pathway and the important role of CaMKK β in Ca^{+2}-mediated cellular processes.

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